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| 14. ABSTRACT The recurrence of prostate cancer is mainly due to the transition of initially androgen-dependent cellular proliferation to androgen-independent growth, or castration-resistant prostate cancers (CRPC). To improve the current therapeutic efficiencies, understanding the mechanisms is critical for developing novel strategies. We found that cell fate determine factor DACH1 regulated AR signaling and androgen-dependent growth. We hypothesis that DACH1/Six1/Eya pathway is an endogenous regulator of AR trans-activation and contributes to prostate tumor onset and progression. New studies demonstrated that DACH1 inhibited the prostate cancer cellular proliferation, not only on AR positive and androgen-independent C4-2 cells but also on AR negative PC-3 and 22RV1 cells. DACH1 inhibited in vitro cellular migration, invasion and tumor growth of PC-3 cells. mRNA microarray analyses and functional validation indicate that repression of IL-6 signaling by DACH1 may be a key molecular mechanism. Inhibitory function of Eya1 on AR transactivation required a phosphates activity and could be enhanced by ectopic expression of co-repressors. Together, our studies show that conserved DACH/Six/Eya pathway regulates hormone signaling and prostate cellular growth. The results may provide a new mechanism for controlling AR signaling transduction and prostate tumorigenesis. | | | | | |
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Introduction

Prostate cancer is the most frequent malignancy and the second leading cause of cancer-related death among men in the United States (1). Based on the unique characteristics that prostate epitheliums are dependent on androgen for growth and survival, androgen deprivation therapy (ADT) has been a first choice of prostate cancer treatment (2). Unfortunately, ADT eventually fails leading to recurrent tumor growth (3, 4); therefore, multiple targets are necessary for efficient therapy. We identified a new member of androgen receptor (AR) co-regulators named *dachshund* (*dac*). *dac* was originally discovered as a dominant inhibitor of hyperactive growth factor receptors in genetic screens (5) and a key member of RDGN pathway, consisting of the *dachshund* (*dac*), *eyes absent* (*eya*), *eyeless*, and *sine oculis* (*so*) (*Six*) genes (6, 7), which is essential for the development of many tissues and organs, such as retinal, ear, kidney, and limb development on mouse (8-12). The close relationships between abnormal expression of RDGN and tumor initiation and progress have been noticed in breast cancer, sarcoma, and other cancer types. We and other have shown DACH1 suppresses human cancer proliferation, migration, invasion and metastasis through different mechanisms (13-18). The DACH1 protein inhibits breast cancer cellular metastasis via the transcriptional repression of IL-8 (19). Breast tumor initiating cells (BTIC) are inhibited by endogenous DACH1 expression through binding to the promoter-regulatory regions of the Nanog and SOX2 genes (20). In gloma cells, homozygously deletion of DACH1 leads to the activation of FGF2 and expansion of tumor initiated cells (21). Most recently, genomic screen in non-small cell lung cancer suggests that mutation of DACH1 may be a novel oncogenic signaling (22). Six1 is overexpressed in breast and lung cancer (23-25) and Six1 overexpression promotes cell cycle progression and transforms breast epithelium and enhances growth of breast tumors in nude mice (26, 27). Six1 up-regulates VEGF-C expression and enhances metastases of rhabdomyosarcoma and breast tumors (28, 29). Six1 induce EMT though increasing TGF-beta signaling or activation of ZEB1 (30, 31). Interestingly, we found that DACH1 negatively regulate TGF-beta signaling (32). Six1 regulates normal stem cell renewal during muscle regeneration (33) and breast cancer stem cells required Eya2 (34, 35). EYA2 is up regulated in ovarian cancer and nerve sheath tumors, promoting tumor growth (36, 37). Further study showed that EYA1 phosphatase is required for promoting tumor cell migration, invasion, and transformation (38). EYA1 and EYA2 enhance survival in response to DNA damage producing agents in HEK293 cells (39). Key members (DACH/Eya/Six) of RDGN coordinately regulate growth and differentiation in response to environmental signaling, unbalanced expressions of each protein cause abnormal organ development and human diseases.

Dach/Six/Eya pathway is required for normal development of genital disc in *drosophila* and prostate gland in mammalian. We previously reported that DACH1 regulates hormone signaling (15, 16); Expression of DACH1 is lost in human prostate cancer tissues and restoration of DACH1 inhibited ligand induced AR activity (15). Whole genomic expression profiles demonstrated decreased DACH1 in prostate carcinoma. This tendency was even stronger in hormone refractory metastatic cancer (40). Significantly high expression of SIX1 was detected in prostate adenocarcinoma, and it positively correlated with metastasis (41). Eya1 was dramatically decreased in prostate carcinoma compared with normal prostate (42). The biological significance and molecular mechanism underlying the irregular RDGN expression in human prostate tumor genesis remain unknown. We hypothesize that RDGN integrates with androgen receptor signaling and altered activation of DACH1/Eya1/Six1 contributes to prostate tumor onset and progression.

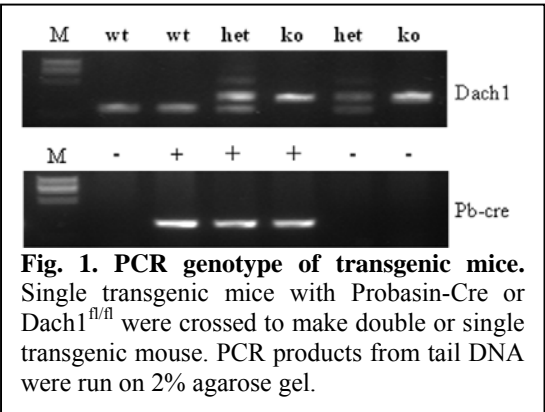
We will: **1)** Evaluate the physiological role of DACH1 in an ErbB2 induced prostate tumor model; **2)** Examine the role of DACH1 in prostate cancer cell AR signaling transduction, proliferation, migration and invasion *in vitro*; **3)** Investigate the role of DACH1 in tumor growth *in vivo* using xenograft models, and **4)** evaluate the clinical significance of DACH1/Eya/Six1 in human prostate cancer.

Collectively, these studies will determine the role of DACH1/Eya1/Six1 pathway in AR signaling transduction, prostate gland development, and prostate tumor onset and progression.

Research Progress:

1. Generation of Prostate Specific Transgenic Mouse at the Thomas Jefferson University.

We acquired *dach1*^{fl/fl} mouse from Dr. Mardon in Bayer College of Medicine. Pb-cre mouse was purchased from NCI-Frederick, strain: b6.Cg-Tg (Pbsn-cre) 4Prb. Mouse tail DNA was extracted using high salt lyses buffer and ethanol precipitation. 100ng DNA was used for PCR amplification. For genotyping PB-cre mouse, PCR primers are: Forward: 5'-ctg aag aat ggg aca ggc att-3'; Reverse: 5'-cat cac tcg ttg cat cga cc-3'. PCR cycling: 94C, 3', 94C 30'', 60C 30'', 72C 30'', 72C 3', 35 cycles. Positive size of PCR product is 393bp. PCR primer for genotyping *dach1*^{fllox} mouse are: forward: 5'-gac cag aac tcc atc cca act-3'; reverse: 5'-aca aca atg tcc tgg gtg ctt-3'. PCR product size is 400bp for Flox allele and 300 bp for wild type. After several round crossing, we now have 13 males with PB-cre positive and *Dach1*^{fl/fl} homozygous at 1, 2, 3 months, 12 mice with PB-cre positive and *Dach1*^{fl/wt} heterozygous. 2 mice were PB-cre negative and *Dach1*^{fl/fl} homozygous at 2 months. ErbB2 activated the androgen receptor pathway in the absence of ligand and synergized with low levels of androgen to 'superactivate' the AR signaling pathway (43-45). Increased expression and altered distribution of ErbB2 represent early events of prostate cancer and relate to the progression of prostatic adenocarcinomas (46). The probasin promoter targeted expression of *erbB2Δ* caused epithelial neoplasm from 5-12 month old mice (47). We are ready to cross those double transgenic mice with Pb-*erbB2* mouse. These studies will assess the ability of endogenous *Dach1* to inhibit the onset and progression of prostate tumor. Due to the low rate of double positive male mice, we are about 5 months behind the schedule.

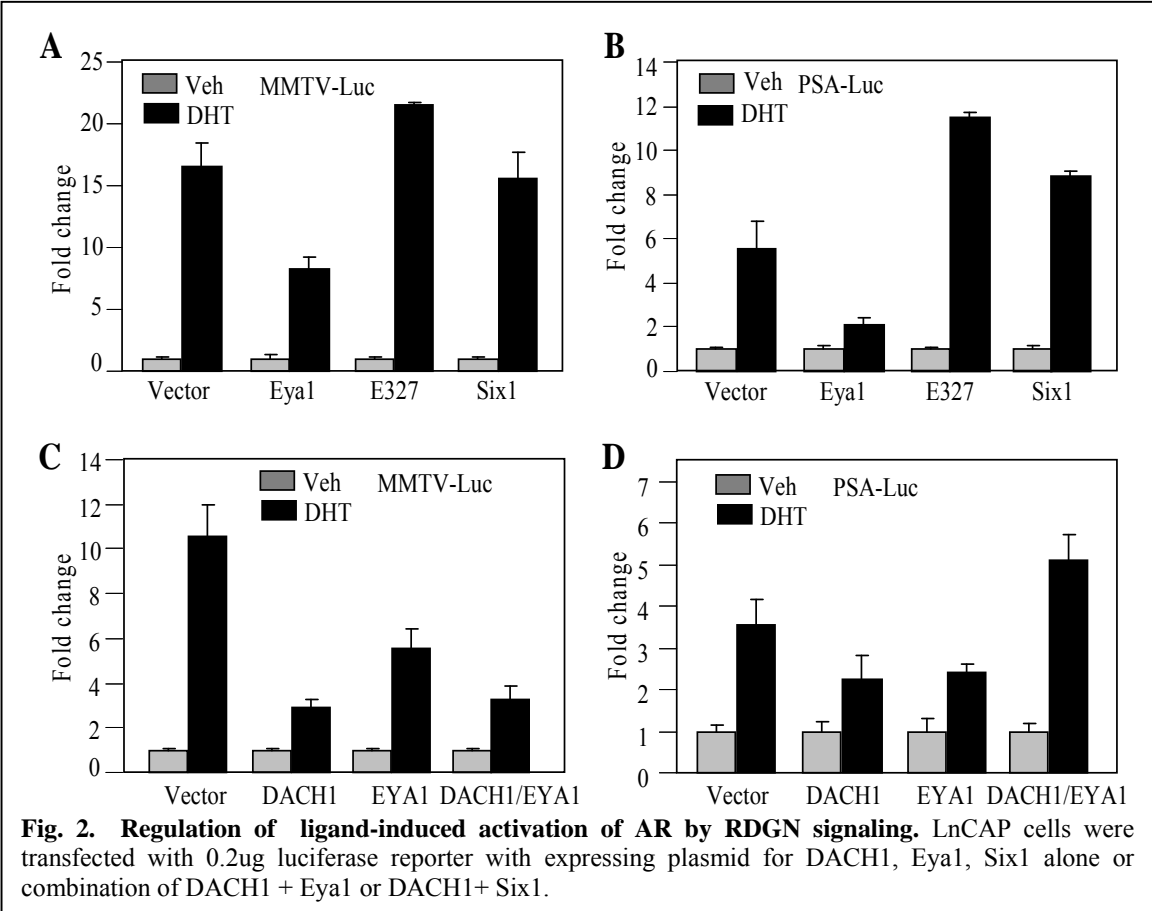


2. In vitro study of DACH1 in AR signaling transduction, prostate cellular proliferation, migration and invasion.

1) Regulation of AR transcriptional activity by DACH1/Eya1/Six1.

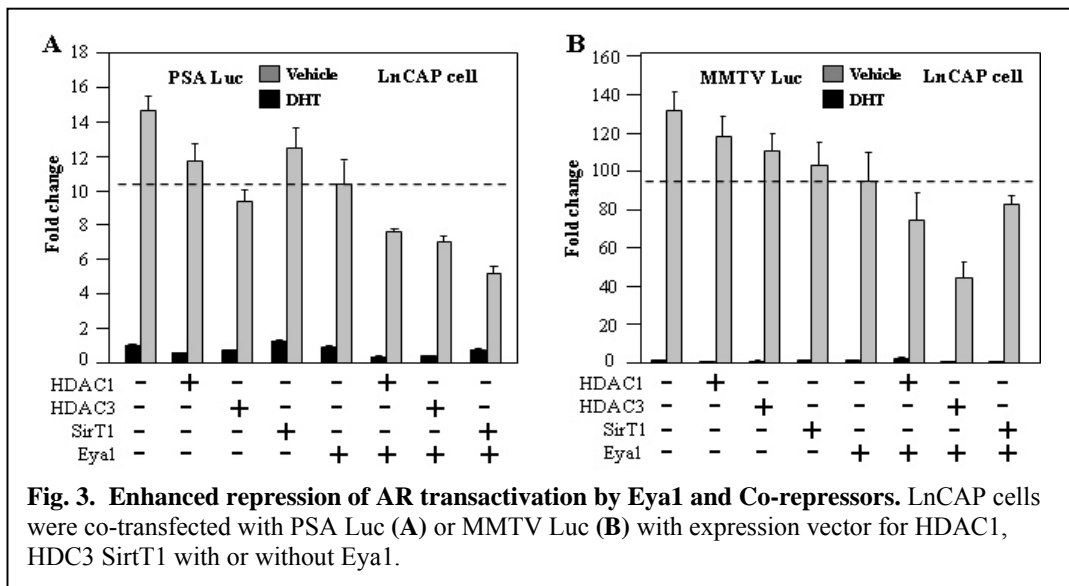
Cell fate determination factor DACH1 integrates androgen signaling transduction through binding with AR and enhanced co-repressor recruitment in local chromatin of AR responsive genes (15). Our previous study also showed that repression of AR trans-activation by

DACH1 is dependent on the relative abundance of co-repressor and co-activator. Repression function of DACH1 could be partly rescued by over-expression of co-activator p300 and SRC, or enhanced by ectopic expression of co-repressor HDACs. Since DACH1, Eya1 and Six1 work together as a network to control cellular differentiation and organ formation during development. We examine whether Six1 or Eya1, key components of the RDGN pathway, could regulate androgen signaling alone or modify activity of DACH1. In hormone



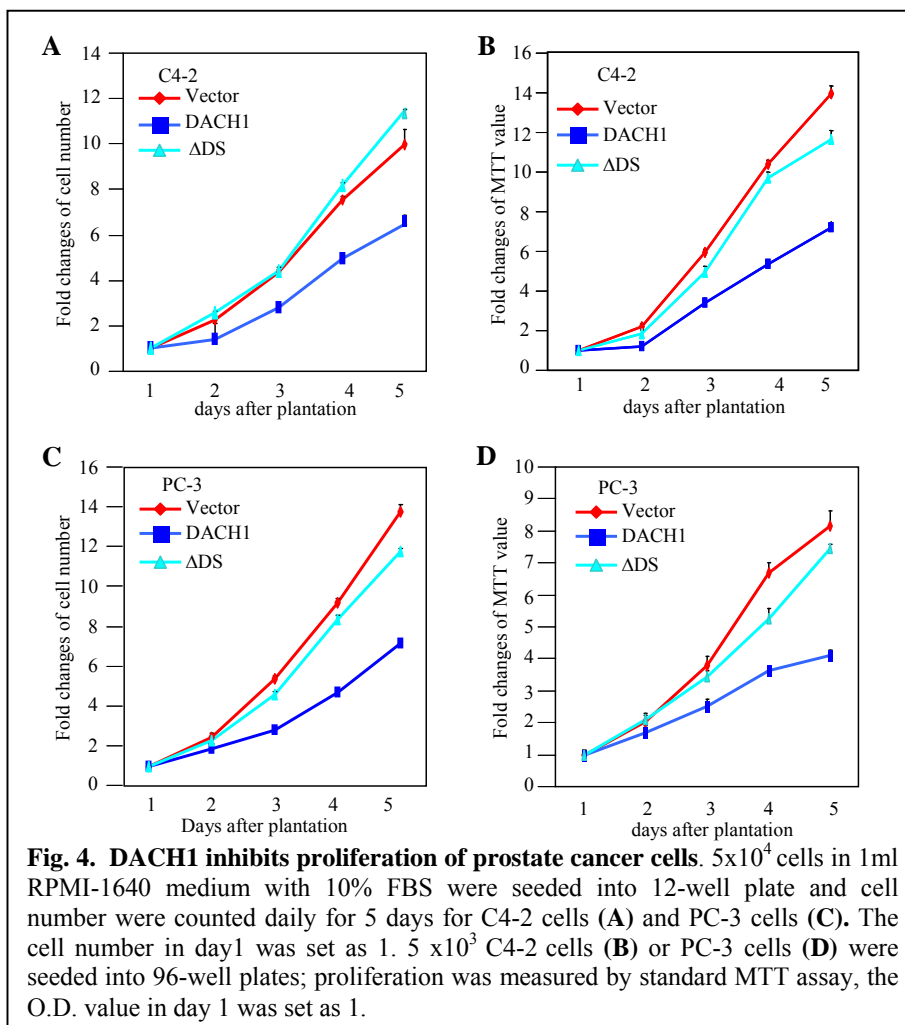
responsive LnCAP cells, ligand stimulation (DHT at 50nM) induced 17 folds activation of MMTV Luc, co-expression of Eya1 reduced transactivation, while phosphatase defective mutant (Eya1D327) is defective in repressive function. Expression of Six1 has no significant effect on ligand-induced activity of AR (Fig. 2A). Similar results were observed in PSA Luc (Fig. 2B). In consistency with

our previous publication (15), DACH1 repressed ligand-induced activation more than 70%, co-expression of Eya1 with DACH1 demonstrated similar function to either DACH1 or Eya1 alone in MMTV Luc (Fig. 2C), but enhanced AR activation in PSA Luc (Fig. 2D), we think the altered recruitment of co-activator or co-repressor may be a molecular mechanism (8). In support with our hypothesis, expressions of AR co-repressors (48-50), HDAC1, HDAC3 or Sirt1 inhibit the ligand-induced AR activation at different degree, co-expression of those inhibitors with Eya1 lead to enhanced repression in both PSA promoter (Fig. 3A) and MMTV promoter (Fig. 3 B). We will test whether co-activators, such as p300, pCAF, or ARA70 could reverse the inhibitory function of Eya1 (51).



2) DACH1 is a broad inhibitor of prostate cancer cellular proliferation *in vitro*.

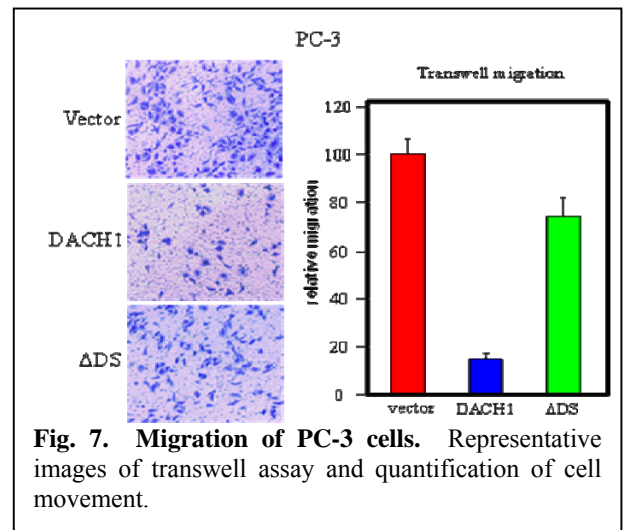
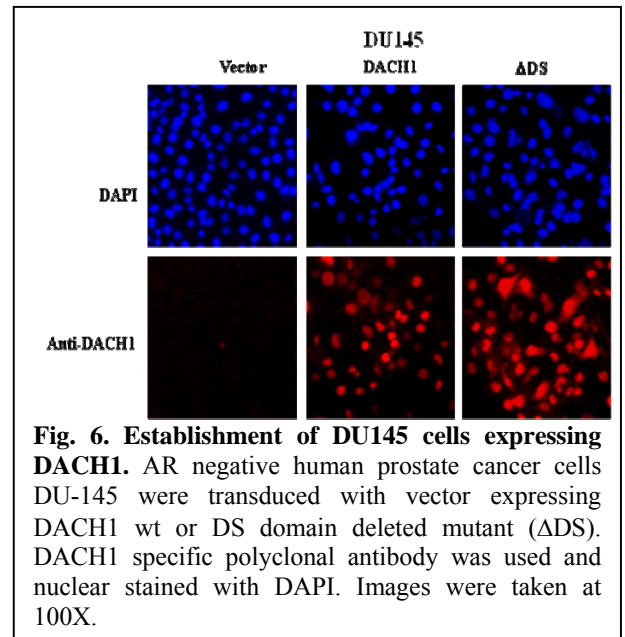
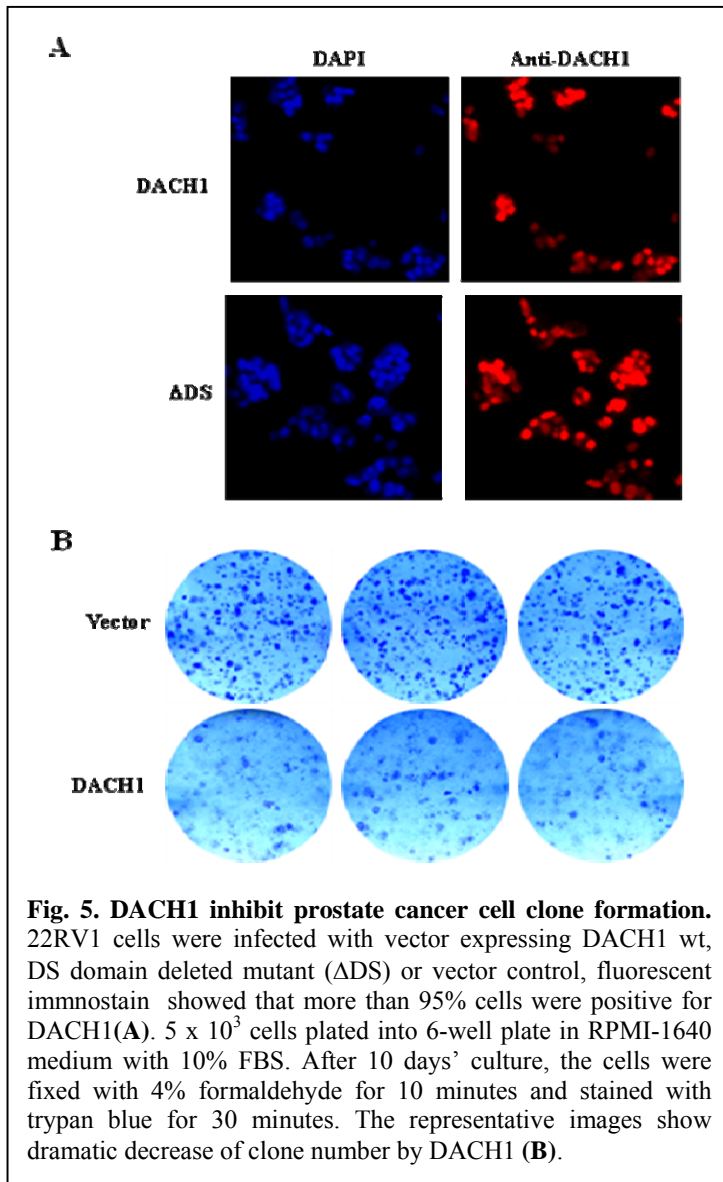
Our previous study showed that DACH1 inhibited proliferation in androgen-dependent LnCAP cells (15). To further explore the significance of DACH1 in human prostate cancer, we established stable cell lines expressing DACH1 or its DS domain deleted mutant (Δ DS) in androgen-independent C4-2 cells and AR-negative PC-3 cells by retrovirus infection. Stable



expressions of DACH1 in more than 90% cells were confirmed by fluorescent immunostain with DACH1 antibody (data not shown). Proliferative ability was evaluated by MTT assay and daily cell number counting. The results show that wild type DACH1 inhibits cellular growth in both cell lines; however, DS domain deleted mutant (Δ DS) has no significant effect on cellular proliferation (Fig. 4A-D).

Prostate cancers progress from androgen responsive to androgen-depletion therapy resistance is major cause of treatment failures, searching for novel therapies targeting those patient is a major clinical challenge. We established a DACH1 stable subline in androgen-independent 22RV1 cells. DACH1 protein expressions were evidenced by anti-DACH1 stain (Fig. 5A). Ectopic expression of DACH1 drastically inhibited clone formation (Fig. 5B).

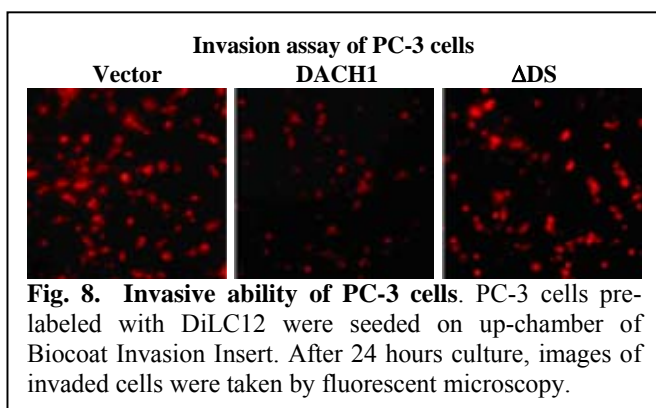
We have also established AR-negative DU-145 sublines stably expressing DACH1 wt and mutant (Fig. 6), further functional study, such as MTT assay, cell number count and clone formation will be performed to evaluate whether DCCH1 has inhibitory effect.



3) DACH1 inhibited migration and invasion of prostate cancer cells.

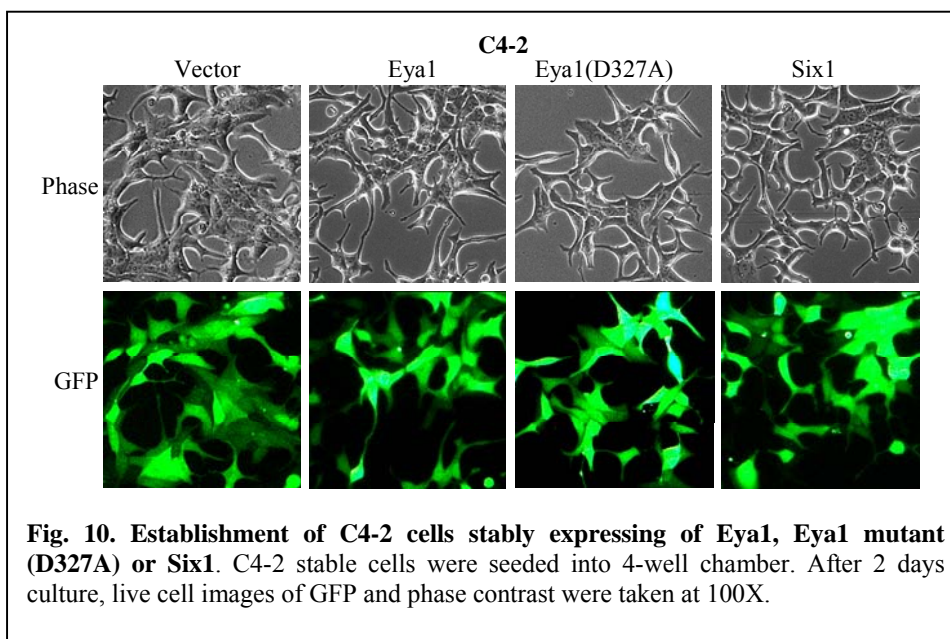
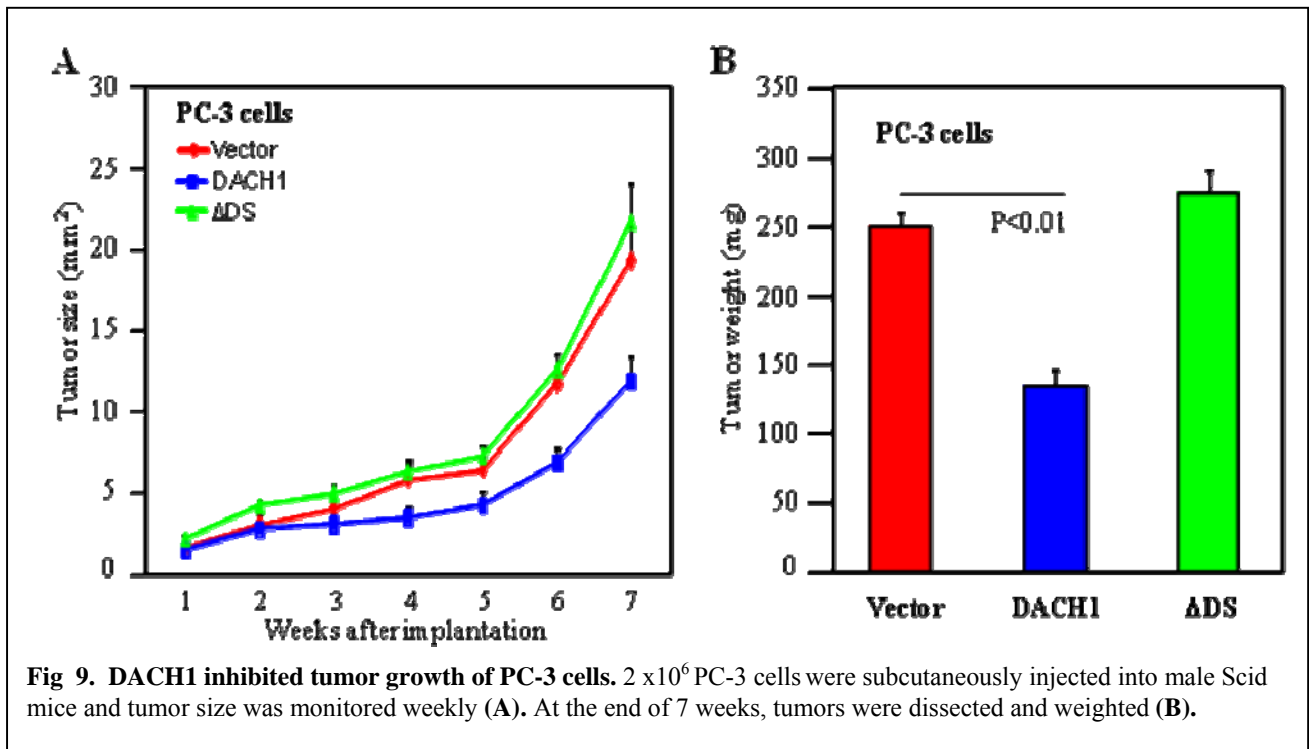
Migration assay was performed using permeable support (Costar, Cat: 3422). After equilibrium 8.0 μ M pore size transwell insert for 1 hour, 5×10^4 PC-3 cells were seeded into up-chamber and cultured for 12 hours. The insert was stained with crystal violet for 30 minutes and cells on up-chamber were removed with cotton swab. 5 fields were randomly selected for counting the migrated cells. The results indicated remarkable inhibition of transmembrane movement of PC-3 cells by expression of wild type DACH1 protein, not by DS domain deleted mutant (Δ DS) (Fig.7).

Invasion assay was performed using BD Biocoat Tumor Invasion System. BD biocoat transwell invasion systems provide cells conditions that allow assessment of the cell invasive property in vitro. FluoroBlok insert with 8 μ M pore size PET membrane that have been uniformly coated with BD matrigel matrix. This uniform layer of BD matrigel matrix serves as are reconstitute basement membrane in vitro providing a barrier to non-invasive cells. Quantization of cell invasion I achieved by pre-cell labeling with Fluorescent dye DiLC12. Since the BD Fluoroblok membrane efficiently block the pass of light from 490-700nm at > 99% efficiency, fluorescent labeled cells that have not invaded are not detected by invert microscopy. The results showed that DACH1 inhibited PC-3 cells invasion at more that 60%, DS domain was required for this function (Fig. 8).



4. *In vivo* study of DACH1 in prostate cancer tumor growth in nude mice.

1) **Tumor growth of PC-3 cells.** A). Cell Lines: PC-3 cells stably expressing DACH1 wild type protein (DACH1), DS domain deleted mutant (Δ DS) or control (vector). B). Mouse: male, 4-5 weeks Scid mice were purchased from NCI-Frederick. C) Group: 10 mice in each group. D) Injection: 2×10^6 cells in 0.1 ml PBS were subcutaneously injected into flank. E) Tumor monitor: weekly measurement was performed using caliper. F) Sacrifice: At the end of 7 weeks, all mice were sacrificed by CO₂ inhalation. G) Tumor tissues: tumors were isolated from surrounding tissues and weighed. H) Histology study: 5 tumors from each group were selected for H.E stain and immuno-histological stain for DACH1 expression. Animal study showed that tumor growths were inhibited by wild type DACH1 to more than 50%, evaluated by either tumor size or tumor weight (Fig. 9), but not by mutant DACH1. Those observations are in consistence with our previous study in breast cancer that DS domain plays a key role in tumor suppressor.



2) **C4-2 cells stably express Eya1, Eya1 phosphatase mutant (D327A) or Six1.** HEK293T cells were transfected with

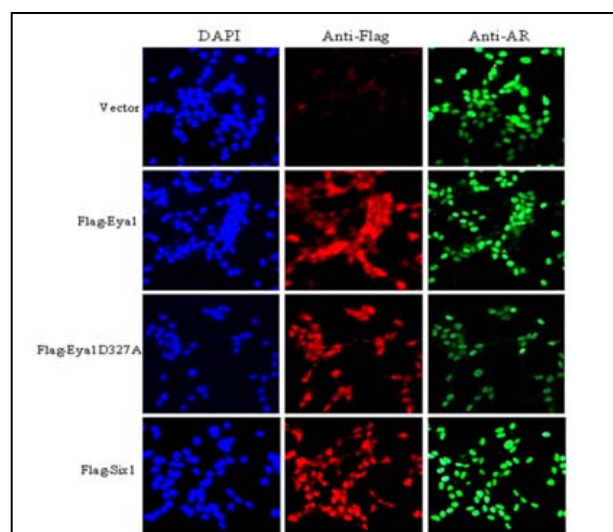


Fig. 11. Expression of Eya1, Six1 and AR in C4-2 cell lines. C4-2 cells transduced with lentivirus vector expressing flag-tagged Eya1 wt, D327A mutant or flag-tagged Six1 were seeded into 4-well chamber. Fluorescent stain was performed with anti-Flag (M2, Sigma) and AR (H280, Santa Cruz). Nuclears were countstained with DAPI. Images were taken at 100X.

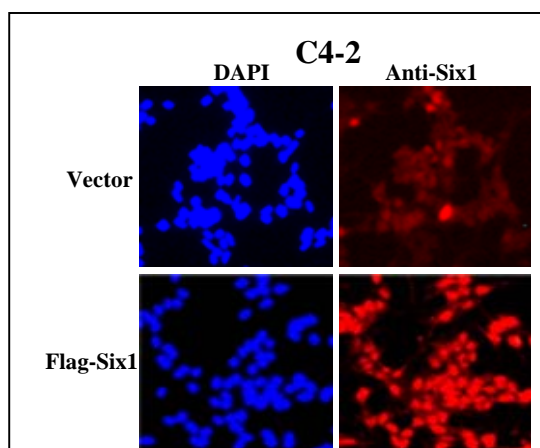


Fig. 12. Enhanced expression of Six1 in C4-2 stable cells. Six1 specific polyclonal antibody from Sigma was used to detect expression in vector cells and Six1 stable cells. Nuclear was stained with DAPI.

ectopic Flag-tag and endogenous AR, nuclear was stained with DAPI. The results indicated stable expressions of Eya1, D327A or Six1; it looks like that the abundance of endogenous AR was inhibited by Eya1 mutant (D327A), not dramatically affected by Eya1 or Six1 (Fig. 11). But to make a scientific conclusion, we are going to perform western blot to quantify the relative expression of AR. We also assessed Six1 expression by using anti-Six1 antibody (HPA001893, Sigma), the images demonstrated about 4-fold enhanced Six1 expression in comparison with vector control cells, which have endogenous Six1 (Fig. 12). Unfortunately, we have not found efficient Eya1 antibody.

In vivo study: prostate cancer cells implantation: As original planed, effects of Eya1 and Six1 on tumor growth are currently tested. Male, 4-6 weeks Nod-Scid mice were purchased from NCI-Fredrick and maintained in transgenic mouse facility of Kimmel Cancer Center. 20 mice were divided into 4 groups: Vector, Eya1, D327A, and Six1. Each mouse received subcutaneous injection of 3×10^6 cells in 0.10ml serum-free medium with 10% matrigel. Currently, mice are 2 weeks after injection. We are monitoring tumor growth weekly. If either Eya1 or Six1 promotes tumor tumor initiation or progression, we will introduce DACH1 into these cells to create Eya1 + DACH1, Six1 + DACH1 double stable sublines. A model hypothesized in previous Nature paper (8) suggested that Eya1 might switch DACH1/Six1 complex from transcriptional repression to activation. Gene expression database indicated that during prostate caner progression to metastatic and hormone refractory stage, expression of DACH1 was lost, meanwhile expression of Six1 was increased.

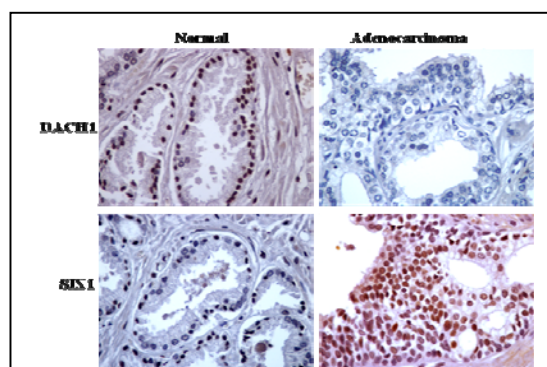


Fig. 13. Expression of DACH1 and Six in normal and tumor tissues (200x).

5. Analyze the expression of DACH1, Eya1 and Six1 in human prostate tumor samples.

Human prostate tissue array consist of 12 normal and 24 tumor samples were purchased from Biomax. Immuno-histochemical stains were performed by a histologist at the Pathological Core Facility of Kimmel Cancer Center. Rabbit polyclone antibody to human DACH1 was purchased from Proteintech. Rabbit polyclone antibody to Six1 was purchased Sigma. Primary evaluation of immune intensity indicated decreased expression of DACH1 with increased protein abundance of Six1 in tumor tissues in comparison with normal prostate gland (Fig. 13). We have tried Eya1 antibodies from several companies, but we have not gotten high-quality results.

6. Target therapy exploiting DACH1-AR interaction.

1). DACH1 antagonist IL6 signaling. To explore the molecular mechanism, we isolated mRNA from PC-3 cells expressing DACH1 wt, DS mutant or vector control and quantitatively measured the RNA expression by microarray. The results indicated that a series of growth and survival factors were repressed by DACH1, such as IL-6, IL-8, CXCL1, CXCL2, CXCL5 and EGF (Appendices). IL-8 is undetectable in AR-response prostate cancer LNCaP cells, but is highly expressed in AR-independent metastatic PC-3 cells. The increased migration, invasion and microvessel density was found in tumor cells expressing IL-8 (52). Increased mRNA expression of IL-8 was associated with both the Gleason score and pathologic stage of tumors and distinguished organ-confined from non-confined tumors (53, 55). Hypoxia increased IL-8 secretion and IL-8 receptor expression in prostate cancer through HIF-1 and NF- κ B signaling (56). Inhibition of IL-8 signaling potentiates etoposide-induced cell death in hypoxic prostate PC-3 cells (56). Therefore, inhibiting IL-8 signaling is recognized as a novel strategy compared to conventional treatment. Since we have previously reported that DACH1 inhibited migration, invasion and lung metastasis of breast cancer cell MDA-MB-231 through decreased secretion of IL-8(19), we now focus on IL-6. IL-6 is a growth and survival factor in human prostate cancer (PCa) cells with aggressive phenotypes and has been implicated in the progression of hormone refractory prostate cancer through multiple mechanisms: **1)** IL-6 activates androgen receptor through increasing of intracrine androgens by enhanced expression of genes mediating androgen metabolism in prostate cancer cells, therefore may promote growth of androgen receptor-positive tumours in vitro and in vivo (57, 58); **2)** IL-6 triggered PI3K/Akt and MAPK/Erk signaling. We identified the A-type cyclin, cyclin A1 as an important downstream target of PI3K/Akt (59); **3)** autocrine IL-6 loop increases cellular levels of antiapoptotic protein and is responsible for therapeutic resistance(60); **4)** IL-6 is sufficient to convert non-cancer stem cell to cancer stem cell in genetically different breast cell lines and a prostate cell line (61); **5)** IL-6

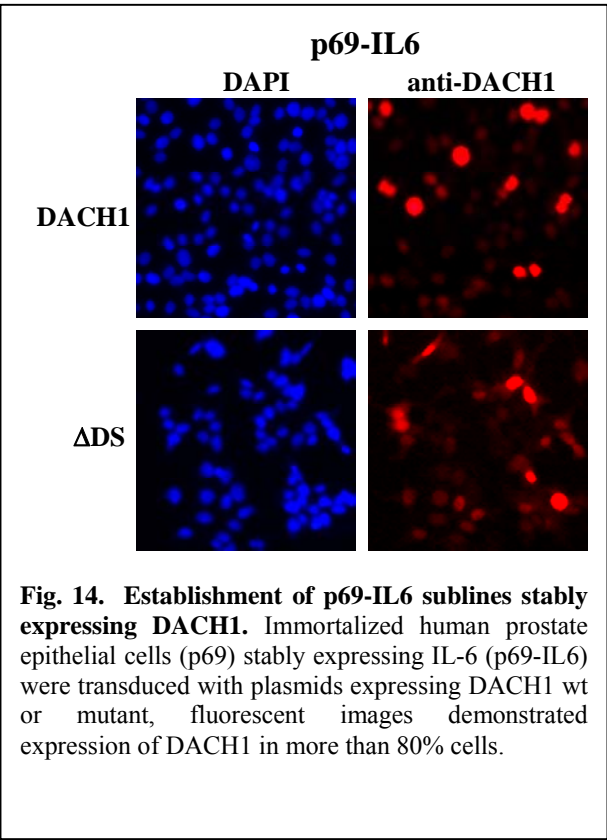


Fig. 14. Establishment of p69-IL6 sublines stably expressing DACH1. Immortalized human prostate epithelial cells (p69) stably expressing IL-6 (p69-IL6) were transduced with plasmids expressing DACH1 wt or mutant, fluorescent images demonstrated expression of DACH1 in more than 80% cells.

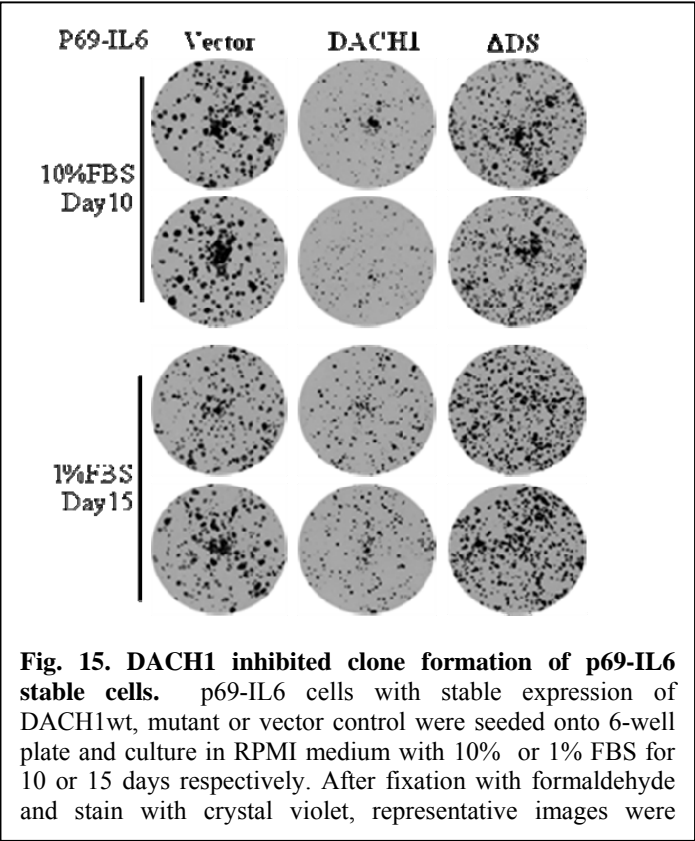


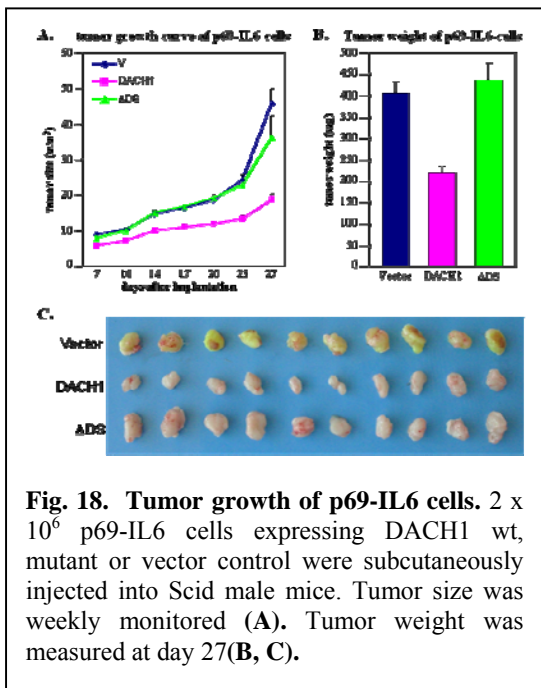
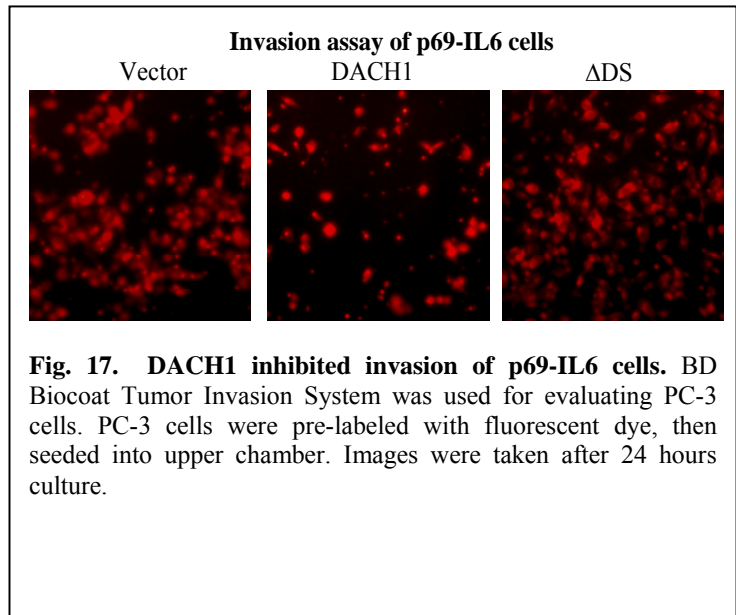
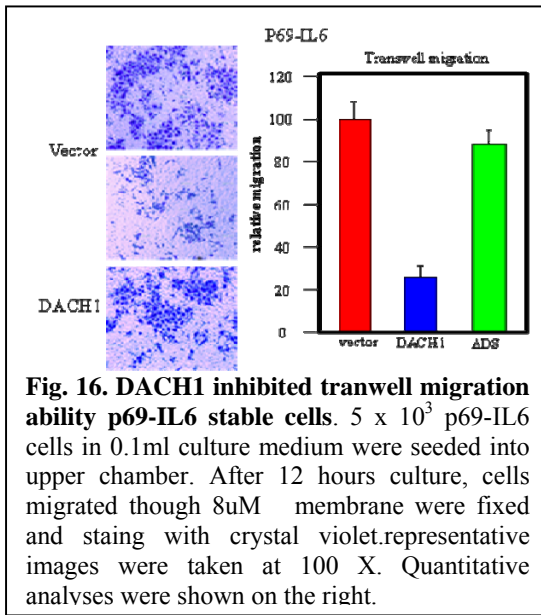
Fig. 15. DACH1 inhibited clone formation of p69-IL6 stable cells. p69-IL6 cells with stable expression of DACH1wt, mutant or vector control were seeded onto 6-well plate and culture in RPMI medium with 10% or 1% FBS for 10 or 15 days respectively. After fixation with formaldehyde and stain with crystal violet, representative images were

can induce tumorigenic conversion and further progression to an invasive phenotype of non-tumorigenic benign prostate epithelial cells (62).

To further analyze the role of DACH1 in IL-6 signaling, we transduced DACH1 into IL-6 immortalized human prostate epithelial cells P69-IL6. Fluorescent stain conformed the stable expression of DACH1 (Fig. 14). Clone formation was inhibited by DACH1 in both 10% FBS or 1% FBS culture condition (Fig. 15).

IL-6 induced migration and invasion were significantly repressed by transwell assay (Fig. 16) and matrigel tumor invasive assay (Fig. 17).

Finally, we tested the role of DACH1 in IL-6 induced *in vivo* tumor growth. Consiting with previous publication, Ther was no tumor formation in untransformed vector controlled p69 cell. Expression of DACH1 delayed tumor formation and inhibited growth ability as avaluated by tumor size and tumor weight, required DS domain (fig. 18)



In therapy-resistant and metastatic prostate cancer, increased levels of IL-6 and IL-8 accompany with decreased expression of DACH1. Ectopic expression of DACH1 in PC-3 cells dramatically inhibited mRNA expression of IL-6 and IL-6 induced proliferation, migration, invasion and tumor growth. It is highly likely that loss expression of DACH1 in prostate cancer attributes to its malignant phenotype partly through paracrine signaling. Our findings suggest that DACH1 may represent potential targets for treatment of prostate cancer.

Key Research Accomplishments

1. DACH1 is a potential prostate tumor inhibitor, not only on AR positive and androgen-responsive LnCAP cells, but also on AR positive, androgen-independent C4-2 and 22RV1 cells;
2. DACH1 inhibited cellular proliferation, clone formation, migration and invasion of AR negative PC-3 cells.
3. DACH1 inhibited tumor growth of AR negative PC-3 cells, requiring DS domain.
4. mRNA microarray analyses of metastatic human prostate cancer cell line PC-3 demonstrated that cell cycle related genes, such as cyclin A, Cyclin E, cyclin D and CDK4 were dramatically inhibited by DACH1 wt, not DS domain deleted mutant.
5. Inhibitory function of migration and invasion in PC-3 cells by DACH1 is through decreased expression of cyterkines/chemokines, such IL6, IL-8 are inhibited by DACH1
6. Eya1 inhibits ligand (DHT)-induced transcriptional activation of AR, required phosphatase function.
7. Examinations of clinical samples indicate increased expression of Six1 during prostate cancer progression.
8. We have aquired double transgenic mice: probasin-CRE/Dach1^{fl/fl} for future *in vivo* study.
9. We have established stable cell lines expressing Eya1, Eya1 mutant, or Six1 to evaluate RDGN pathway in hormone signaling transduction and tumor growth.

Reportable Outcomes:

1. Prostate specific conditional double transgenic mouse model: Pb-cre/Dach1 fl/fl, which is very valuable for study the endogenous Dach1 in prostate gland development, since dach1 knock out mouse can not survival more than 2 days. If crossed with prostate oncogenes, this mouse will be good model to determine the role of Dach1 in prostate tumor formation.
2. Gene expression profiles of prostate cancer cells with or without DACH1 can be used for pathways analyses to help identify molecular mechanism in prostate cancer as well as in other cancer types.
3. Development of several cell lines stably expressing key components of RDGN pathway: Eya, Dach1 and Six1. In vitro and in vivo studies of those cell lines promote better understanding of AR signaling.
4. Abstract will be submitted to 2013 AACR annual meeting.

Conclusion:

RDGN network integrates with androgen receptor signaling. Inhibition of prostate cancer cellular proliferation is through AR-dependent or independent pathway. DS domain of DACH1 protein, which consists of 100 amino acids, plays a key role in tumor suppression and represents a potential therapeutic target. Eya1 represses androgen receptor activation, but its role in prostate cellular growth and in vivo tumor formation remains to be elucidated.

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Appendix

Relative expressions of selected gene in PC-3 cells

